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Functional and Structural Evaluation of Bovine Heart Cytochrome *c* Oxidase Incorporated into Bicelles[‡]

Andrey Musatov^{a,✉}, Katarina Siposova^a, Martina Kubovcikova^a, Veronika Lysakova^{a,b}, and Rastislav Varhac^b

^aInstitute of Experimental Physics, Slovak Academy of Sciences, Watsonova 47, 040 01 Kosice, Slovakia

^bDepartment of Biochemistry, Institute of Chemistry, P. J. Safarik University in Kosice, Moyzesova 11, 040 01 Kosice, Slovakia

Abstract

Bilayered long- and short-chain phospholipid assemblies, known as bicelles, have been widely used as model membranes in biological studies. However, to date, there has been no demonstration of structural or functional viability for the fundamental mitochondrial electron transport complexes reconstituted into or interacting with bicelles. In the present work, bicelles were formed from the mixture of long- and short-chain phospholipids, specifically 14:0 and 6:0 phosphatidylcholines (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine, (DHPC)). Isolated from bovine heart, cytochrome *c* oxidase was successfully incorporated into bicelles. Bicelles and cytochrome *c* oxidase incorporated into bicelles (“proteobicelles”) were characterized by absorption spectroscopy, dynamic light scattering, atomic force microscopy, sedimentation velocity and differential scanning calorimetry. It was demonstrated that at total concentration of phospholipids $C_L = 24$ mM and the molar ratio (*q*) of long-chain DMPC over short-chain DHPC equal to 0.4, the diameter of bicelles formed at neutral pH is in the range of 30 – 60 nm with the thickness of bicelles of about 4 nm. Adding cytochrome *c* oxidase to bicelles unified the size of the resulting proteobicelles to about 160 nm. Cytochrome *c* oxidase in bicelles was fully reducible by artificial donors of electrons, exhibited “normal” reaction with external ligands, and was fully active. Both, sedimentation velocity analysis and temperature-induced denaturation indicated that enzyme in bicelles is monomeric. We concluded that cytochrome *c* oxidase in bicelles maintains its structural and functional integrity, and that bicelles can be used for more comprehensive investigation of cytochrome *c* oxidase and most likely other mitochondrial electron transfer complexes.

Graphical Abstract

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[✉]Corresponding author: Institute of Experimental Physics, Slovak Academy of Sciences, Watsonova 47, Kosice, Slovakia; Tel: +421 55 720 4132; Fax: +421 55 633 6292; musatov@saske.sk.

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Keywords

cytochrome *c* oxidase; bicelles; DHPC; DMPC; detergents; aggregation state

Introduction

Cytochrome *c* oxidase (EC 1.9.3.1; Complex IV, CcO) is a large transmembrane protein-phospholipid complex found in bacteria and mitochondria of eukaryotes. This multi-subunit enzyme catalyzes the four-electron reduction of dioxygen to water, a reaction coupled to proton translocation across the inner mitochondrial membrane [1]. Mammalian CcO is consisting of 13 dissimilar subunits, three or four tightly bound phospholipids, and four metal centers (Cu_A , heme *a*, heme *a*₃, and Cu_B) with a combined molecular weight of ~ 205 000 for the monomeric enzyme [2]. Mitochondrial proteins represent an important class of membrane proteins that remain especially difficult to study. One feature that most directly impacts structural and functional studies of this multi-subunit protein-lipid complex *in vitro* is its high hydrophobicity. For example, mitochondrially encoded CcO subunits I, II and III are very hydrophobic and have Gravy hydropathy scores of 0.685, 0.247 and 0.413, respectively [3, 4]. To overcome issues due to the amphiphilic character of CcO, different strategies have been used, such as solubilization with different single detergents [5, 6], mixture of detergents [7], or incorporation of enzyme into phospholipids multilamellar or unilamellar vesicles [8, 9]. Although detergents are capable of effectively solubilizing such hydrophobic proteins, they cannot mimic the interactions between proteins and lipid membranes, one of the essential features of cell operation. Indeed, numbers of biochemical and biophysical studies have demonstrated that the lipid bilayer is important for structural and functional integrity of membrane proteins. Therefore, reconstitution of membrane proteins into phospholipid-based bilayer (liposomes) is one of the most useful techniques to study the structure and function of these proteins. In fact, incorporation of CcO into liposomes was successfully used for the examination of proton pumping mechanism, ligand binding, etc. [8,9,10]. However, the liposomal turbidity does not permit the use of some of the experimental techniques, such as absorbance spectroscopy or circular dichroism. This issue can potentially be overcome by finding new methodology for biophysical, enzymatic or structural investigations *in vitro*. Such approach was recently applied by Öjemyr et al. [11]. In this work two different terminal oxidases, *aa*₃ from *Rhodobacter sphaeroides* and *ba*₃ from *Thermus thermophilus* were reconstituted into nanodiscs composed of soybean lipids using the scaffold protein.

To probe the structure of membrane proteins in their native environment (i.e., bilayer), a new phospholipid mixture known as “bicelle” has been developed [12,13]. Bicelles are nanostructures formed by a long- and a short-chain phospholipids or detergent dispersed in aqueous solution. In contrast to liposomes, bicelles do not have an aqueous inside and their properties are close to a liquid crystal phase with one or two-dimensional ordering. Moreover, bicelles are transparent and, thus, amenable to optical spectroscopy. To date, the combination of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) as long-chain, bilayer-forming component with 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) as short-chain component (or combination with 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO)) has remained the most popular choice for bicelle formation. The morphology of bilayered lipid mixtures highly depends on total concentration of lipids, ratios between long- and short-chain lipids, lipids composition, presence or absence of monovalent or divalent cations, time of hydration and/or temperature [14,15,16]. Several structural models for bicelles have been reported, such as disc-shaped, cylindrical micelles or perforated lamellae. However, the most recognized structural feature is a disc-shaped structure with the long-chain lipids organizing the disc plane and the short-chain lipids stabilizing the edges (or the rim) of the bicelles. This system is suitable for a number of various applications in different research areas, e.g., solution and solid-state NMR study on membrane proteins [17], crystallization of membrane proteins [18] circular dichroism (CD), and small-angle neutron scattering (SANS) [19,20]. Surprisingly, one very important group of proteins - mitochondrial electron transfer complexes, was completely ignored.

Our present work was designed to investigate the interaction of bicelles formed from the mixture of DMPC and DHPC with isolated mitochondrial electron transfer Complex IV or cytochrome *c* oxidase.

Materials and Methods

Materials

Both, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) in chloroform were obtained from Avanti Polar Lipids, Inc. and used without further purification. Triton X-100, sodium cholate, horse heart cytochrome *c* (95% purity), hydrogen peroxide and sodium dithionite were purchased from Sigma-Aldrich. *n*-Dodecyl β -D-maltoside was obtained from Anatrace. All other chemicals were reagent grade.

Methods

Bovine cytochrome *c* oxidase—Two forms of isolated from bovine heart CcO, designated as A and B forms, were used in this work. Preparation A (slow reacting with the external ligands) was isolated and purified from mitochondrial Keilin-Hartree particles by the method of Fowler et al. [21] as modified by Capaldi and Hayashi [22]. The final enzyme precipitate was solubilized at ~ 150 μ M heme *aa*₃ in 0.1 M NaH₂PO₄ buffer (pH 7.4) containing 25 mM sodium cholate and then frozen in liquid nitrogen. CcO subunit composition was verified by reversed-phase HPLC as described previously [23]. The

cytochrome *c* oxidase concentration was determined from the dithionite reduced minus fully oxidized spectrum and quantified using molar extinction coefficient $\varepsilon_{605-630} = 27 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. The phosphorus content (15–20 P/aa₃) was determined as described previously [24]. Cytochrome *c* oxidase activity was measured spectrophotometrically following the pseudo-first-order rate of oxidation of 25–30 μM ferrocytochrome *c* by 2 nM cytochrome *c* oxidase at pH 7.0 in 25 mM phosphate buffer containing either 2 mM dodecyl maltoside or DMPC/DHPC mixture. Temperature effect on CcO activity was analyzed after solubilized enzyme was heated from 25 °C to 75 °C in water bath at rate of 1.5 °C/min. At 5 °C intervals, a 10 μL aliquot was removed and diluted with 190 μL ice-cold assay buffer, containing 2 mM DM. The activity was analyzed at 25 °C. Preparation B (reacting fast with the external ligands) involves Triton X-100 solubilization of enzyme from mitochondria at neutral pH followed by purification using ion-exchange chromatography in the presence of Triton X-100. The resulting CcO was concentrated to ~ 200 μM and frozen in liquid nitrogen. The phosphorus content (5–10 P/aa₃) was determined after extraction of phospholipids by the method of Bligh and Dyer [25]. Enzyme isolated in TX-100 according to modified protocol of Soulimane and Buse [26, 27] was a gift from Dr. M. Fabian from The Center for Interdisciplinary Bioscience P.J. Safarik University in Kosice, Slovakia.

Formation of bicelles and proteobicelles—The different amount of single phospholipid (DHPC) or mixture of DMPC and DHPC was dried out under stream of nitrogen. The resulting PL film was suspended by brief and gentle vortexing in appropriate buffer for 10–20 sec at room temperature. The hydration of phospholipids and the formation of bicelles was achieved by incubation of PL for about 3 hours at room temperature with an occasional (every 20–30 min) vortexing. The resulting clear bicellular solution was obtained. CcO incorporation to bicelles was accomplished by addition of a few microliters of concentrated detergent-solubilized enzyme (final CcO concentration was ~ 5 μM) to bicelles. All measurements with CcO in bicelles were performed after incubation of protein-phospholipid mixture for 30–60 min at room temperature.

Absorption spectroscopy—All spectra and kinetic measurements were recorded digitally using either a SLM Aminco 3000 or Jasco V-630 diode array spectrophotometer in 1 ml semimicro cuvettes with blackened walls and optical path of 1 cm. Difference spectra of the H₂O₂ or cyanide-induced spectral shifts were calculated by subtracting the oxidized absolute spectra of CcO from the ligand-induced absolute spectra.

Atomic force microscopy—Samples for AFM were prepared by casting an aliquot (5 μL) bicelles solution ($C_L = 24 \text{ mM}$) on a freshly cleaved mica surface (highest grade V1 mica discs Ted Pella). The sample was dried for at least 24 hours in air at room temperature. The images of dried bicelles were obtained using an NCHV cantilever (force constant 42 N/m and nominal resonance frequency 320 kHz and a specific resistance of 0.01–0.025 $\Omega \text{ cm}$, antimony (n) doped Si, a radius of the tip curvature of 10 nm) with Scanning Probe Microscope (Veeco di Innova, Bruker AXS Inc., Madison, WI, USA) working in a tapping mode. The scan rate was 0.25–0.5 kHz and the resolution of image was 512 pixels per line resolution (512×512 pixels/image). No smoothing or noise reduction was applied. The AFM

images were analyzed using NanoScope Analysis1.20 (Veeco di Innova, Bruker AXS Inc., Madison, WI, USA).

Dynamic light scattering—Apparent hydrodynamic diameter of the prepared bicelles and proteobicelles was determined by dynamic light scattering (DLS) using a Zetasizer Nano-ZS from Malvern Instrument. Measurements were performed in small-volume cuvettes with a 1 cm light path. The experimental conditions were: 1 mL of bicelles ($C_L = 2.4$ mM) or proteobicelles (0.5 μ M CcO in bicelles with $C_L = 2.4$ mM) in appropriate buffer at 25 °C. The viscosity and refractive indices of water used for analysis 0.89 centipoises and 1.33, respectively. Samples were illuminated with a laser with a wavelength of 633 nm. The DLS intensities of samples at a 173° scattering angle in kilo counts per second were collected for 15 runs with 20s duration each run and then averaged. To obtain the size distributions the collected data were analyzed using the Non-negative Least Squares (NNLS) method. Hydrodynamic diameter was calculated under the assumption that scattering particles follow to diffusion properties observed for globular particles undergoing Brownian motion.

Differential scanning calorimetry—DSC experiments were performed on a VP-DSC differential scanning microcalorimeter (Microcal, Northampton, MA). The scanning rate was 1.5 °C/min. Protein concentration was always 5 μ M in 20 mM Tris-Cl, pH 8.0 buffer containing either, detergent or bicelles. Before each measurement, the sample and reference solutions were degassed in an evacuated chamber for 5 min at room temperature. A pressure of 2 atm was kept in the cells throughout the heating cycles to prevent degassing. A background scan, collected with buffer in both cells, was subtracted from each sample scan. Excess heat capacity curves were plotted using Origin software supplied by Microcal.

Sedimentation velocity—Sedimentation experiments were performed in the Center for Analytical Ultracentrifugation of Macromolecules located in the Department of Biochemistry at the University of Texas Health Science Center, San Antonio, Texas. Sedimentation velocity studies were carried out in an Optima XL-I Beckman analytical ultracentrifuge. The rotor speed was 40 000 rpm. Data were collected at 20 °C using optical absorption detection at 420 nm. The scans were analyzed by the method of van Holde-Weischet [28], using the UltraScan II version 9.3 data analysis software developed by B. Demeler (Department of Biochemistry, University of Texas Health Sciences Center). Use of this program has been described previously [29] and is available at <http://www.cauma.uthscsa.edu>. The data were corrected for buffer density and viscosity. The contribution of phospholipids to the effective mass was ignored since the partial specific volume of phospholipids is close to zero. However the detergent binding is unknown. Because of this uncertainty, the sedimentation velocity data are presented as S_{obs} (observed sedimentation coefficient). The initial CcO concentration for sedimentation velocity experiments was 5 μ M. Monomeric CcO either in 10 mM Triton X-100 or 10 mM DM was prepared using protocol described previously [7].

Results and discussion

Phospholipids ratio screening

Different concentrations of DMPC and DHPC, but also single short-chain PL (DHPC) were tested in order to choose the “best experimental” conditions for solubilization of isolated CcO. In each case the concentrations of PLs were higher than critical micelle concentration (CMC), 15 mM and 6 mM for DHPC and DMPC, respectively. The criteria of the best conditions were the spectral characteristics of both, reduced and oxidized forms, particularly reducibility of CcO heme *a* and heme *a*₃ by artificial donor of electrons, sodium dithionite. Expected absorption spectrum of oxidized CcO was observed in buffer containing 2 mM DM (Figure 1, left panel). The oxidized enzyme exhibited an α absorption band at ~ 599 nm and a γ -band (Soret band) in the region of 420 nm. Reduction with sodium dithionite intensified and shifted the α -band to 605 nm. Short-chain phosphatidylcholine, DHPC is structurally a phospholipid but due to its short fatty acyl chain of 6 C-atoms it exhibits detergent-like properties. Therefore, DHPC at concentration higher than CMC should form micelles and solubilize hydrophobic CcO. To test this hypothesis CcO was added to buffer containing different concentration of DHPC (17 to 44 mM). Spectra of oxidized enzyme in DHPC suggest that CcO is not aggregated in DHPC-containing buffer since the enzyme exhibited the typical absorption spectra (Figure 1, middle panel). However, although sodium dithionite was able to induce spectral red shift, that is characteristic to reduced CcO, the ratio of $A_{444}(\text{reduced})/A_{420}(\text{oxidized})$ for CcO in DM buffer equal to 1.2 was higher than the ratios obtained in DHPC-containing buffer (1.1–1.06) indicating incomplete reduction of the hemes under these specific conditions. The reduction of hemes was neither time- nor dithionite-concentration dependent.

Second set of experiments was performed using the bicelles that were formed from a mixture of DMPC and DHPC. In these experiments spectra of oxidized and dithionite reduced enzymes were recorded after solubilization of 5.1 μM CcO in 50 mM Tris-Cl buffer, pH 7.4 containing DMPC/DHPC mixture (Figure 1, right panel). The total concentration of PL (C_L) was always 24 mM, but DMPC to DHPC ratio (q) was varied from 0.3 to 0.7. The optical spectra of oxidized, reduced, or the ratio of $A_{444}(\text{reduced})/A_{420}(\text{oxidized})$ were very similar for both DM and PL mixture solubilized CcO, suggesting that bicelles are very effective at solubilizing CcO. Only small decrease of reducibility ~ 5% was observed with $q > 0.6$. Both preparation of CcO (preparation A and B) in different buffers in the pH range of 7.0 to 8.0 showed the same results.

Direct visualization of DMPC/DHPC bicelles by AFM

The shape, thickness, size and dispersion of bicelles prepared at neutral pH were characterized by AFM using a tapping mode to minimize possible destructive forces (Figure 2). AFM visualization demonstrated that bicelles are not uniform in sizes. At least two circular-shaped species with different size were observed. It is possible that the observation of circular species by AFM instead of disc-shaped reflects a consequence of water evaporation from the bicelles. However, it is also possible that at relatively high concentration of DHPC and low q the bicelles are circular-shaped. Using a surface profiling line that was drawn across a part of the image and applying the software supplied by Bruker

AXS Inc. the diameter of the most abundant bicelles was measured to be ~ 50 nm (Figure 2, panels C and D). In addition, surface profiling also allowed measuring a height of the bicelles which was found to be 4 ± 1 nm (Figure 2, panel A). It should be noted, that the thickness of the DMPC bilayer (h) has been estimated as about 4 nm [30]. Thus, the AFM images can provide a direct quantitative picture of the isotropically tumbling bicelles assemblies. Moreover, using the thickness measured perpendicular to the scanning plane (Figure 2, panel B) bicelles thickness was also estimated as 3–5 nm, matching exactly the data from surface mapping and a thickness that was expected for the lipid bilayer [15,31]. The diameter of bicelles of about 50 nm is larger than theoretically calculated [32], however is in a range of 30–100 nm as experimentally determined by electron microscopy [16, 33].

Incorporation of CcO to bicelles - dynamic light scattering measurements

The AFM results were fully supported by DLS measurements. Bicelles were prepared by the same way as above at total phospholipid concentration $C_L = 24$ mM. CcO was added to bicelles and DLS analysis performed after about 1 hour incubation at room temperature. Both, pure bicelles and CcO-bicelles mixture were diluted 10-fold with an appropriate buffer before the analysis. Dynamic light scattering experiments revealed that CcO-free phospholipid mixture consist of two major components. The size distribution of bicelles prepared and measured at pH 7.4 at 25 °C consisted of two peaks with an average apparent hydrodynamic diameter of about 15 nm and 58 nm (Figure 3, panel A). The value of 58 nm is in good agreement with the value of ~ 50 nm obtained by AFM. Figure 3 also shows a dynamic light scattering result for CcO mixed with DMPC/DHPC bicelles. Only one single peak with an average apparent hydrodynamic diameter of species ~ 165 nm was detected, suggesting that the main fraction of the CcO - bicelles preparation was uniform having a well-defined size. Therefore, interaction of CcO with bicelles of different sizes resulted in size unification of “proteobicelles”. The hydrodynamic radii of monomeric and dimeric detergent-solubilized CcO were estimated from the sedimentation coefficients and found to be about 66–85 Å, i.e. ~13–17 nm in diameter [34]. Incorporation of such large complex in low q bicelles with diameter of about 50 nm definitely does change the size of the bicelles. For example it was demonstrated that in the presence of K^+ and Na^+ , bicelles have diameters of about 30 nm, but in the presence of Ca^{2+} and Mg^{2+} their diameter is ~ 50 nm [16]. Without knowledge of the amount of bound detergent and/or phospholipid it is impossible to make a conclusion whether CcO is monomeric or dimeric in bicelles. However, the data clearly indicates that CcO is completely reconstituted into bicelles, since no additional peaks were observed.

Probing of reactivity of heme a_3 - reaction with ligands

The reaction of external ligands with CcO proves to be a useful indicator of the “nativity” of the isolated enzyme. The rate and spectral characteristics of the reaction can indicate on possible modifications of heme a_3 or its close surroundings. Two well-known ligands have been used to test heme a_3 reactivity of CcO in bicelles – cyanide and hydrogen peroxide. The reaction of CcO with either cyanide or H_2O_2 is routinely measured by following the changes in absorbance in the Soret and/or α region [35, 36, 37]. CcO reacts with KCN to causes a time dependent red shift in the Soret maximum from 418–423 to 427–428 nm. It is well known that cyanide binds relatively slowly to the cholate-isolated preparation A of

CcO. The monophasic and fast reaction of oxidase with cyanide is typical of the preparation B of CcO as was demonstrated previously [38,39]. Under our experimental conditions, in agreement with previous data for preparation A of CcO two phases of cyanide binding were observed. First, initial fast phase was characterized by the rate constants of 0.013 s^{-1} and 0.0056 s^{-1} for bicelles and DM-solubilized enzymes, respectively. Therefore, changing the solubilizing detergent for bicelles alters the rate constant for the cyanide reaction by as much as 2-fold. However, such effect of solubilizing agent on cyanide reaction is not unusual and similar alterations in the rate constant were described previously by Panda and Robinson [35]. The rate constants of the second, slow phase were 0.00066 s^{-1} and 0.0008 s^{-1} , for bicelles and DM-solubilized enzymes, respectively. These numbers are in a good agreement with the data published for the reaction of CcO with cyanide [40]. Therefore, the reaction of CcO in bicelles with cyanide is similar to the reaction of detergent-solubilized CcO with cyanide. This suggests that heme a_3 of CcO in bicelles is not altered and is fully accessible to external ligand.

The mechanism for O_2 reduction by CcO involves formation of intermediates, including the “peroxy” (P or 607 nm) and the “ferryl” (F or 580 nm) forms [41]. It is generally accepted that CcO species related to these intermediates can be experimentally generated by H_2O_2 [37,41]. We have tested whether CcO in bicelles is capable to react with hydrogen peroxide and produce P and F intermediates. Addition of H_2O_2 to CcO at neutral pH produced the expected absorption spectral changes in the Soret and α -region similar to those previously described for detergent-solubilized CcO [37]. The Soret maximum shifted from an initial value of 420 nm to 427 nm after incubation with 5 mM H_2O_2 for 3 min at room temperature (Figure 4). The difference spectra (H_2O_2 -treated CcO minus untreated CcO) in the Soret region were characterized by typical maximum at 436 and minimum at 412 nm for CcO in bicelles and in DM (Figure 4, inset panel). The α -region was characterized by two maxima, one at 580 nm and the other at 607 nm, consistent with the production of a mixture of the ferryl and peroxy forms. A total concentration of P and F forms was calculated using $\epsilon_{434-412} = 65\text{ mM}^{-1}\cdot\text{cm}^{-1}$ [37] and was found to be 2.5 μM , i.e. 50% of CcO was in P plus F form after the reaction of 5 μM of CcO with H_2O_2 in bicelles. Similar amount of P and F intermediates (about 40% of total amount of the enzyme) was generated by H_2O_2 -treated CcO solubilized in DM. Therefore, the ligand-binding properties of the CcO are not perturbed by incorporation into bicelles, and heme a_3 remains intact.

Electron transfer activity

Cytochrome *c* oxidase activities were measured in 25 mM phosphate buffer, pH 7.2 in presence of either DM or DMPC/DHPC bicelles (the data are summarized in Table 1). Typically the activity of DM-solubilized CcO measured in DM-containing buffer was $\sim 350\text{ s}^{-1}$ (100% activity). However, if DM-solubilized enzyme was analyzed in presence of bicelles the activity was significantly decreased and was in the range of $85\text{--}95\text{ s}^{-1}$ ($\sim 30\%$). Even lower electron transfer rate was detected if the activity of CcO incorporated into bicelles was measured in bicelles-containing buffer ($\sim 20\%$). However, activity of CcO in bicelles was almost fully recovered when this enzyme was analyzed in DM-containing buffer ($\sim 95\%$). The loss of CcO activity in bicelles can be explained by at least three distinct mechanisms. First, DHPC can affect the rate of intramolecular electron transfer

between heme *a* and heme *a*₃ within CcO. Such effect of detergents on intramolecular transfer was demonstrated previously [42,43]. Second, CcO low enzymatic activity in bicelles can be explained by zwitterionic character of both DMPC and DHPC. Although cytochrome *c* is completely dissolved in bicelles and it fully reducible by sodium dithionite (data not shown), zwitterionic PLs can affect an ionic interaction of cytochrome *c* with CcO. Alternatively, the loss of activity can be due to requirement of CcO in a fluid environment of fairly long fatty acid chains [44] and therefore, bicelles do not create a proper hydrophobic environment for activity assay. At present, it is not possible to differentiate between the three possibilities. However it can be concluded that bicelles do not trigger unwanted conformational changes of CcO, since loss of activity is completely reversible.

Homogeneity of CcO in bicelles - sedimentation velocity analysis

The aggregation state of detergent-solubilized CcO is strongly dependent upon both the type and concentration of detergent [29]. At low concentration of a single detergent the preparation A of CcO is often a mixture of monomers and dimers. Increasing of concentration of nonionic non-denaturing detergents, such as DM or TX-100 resulted in monomerization of enzyme, which sediments as a homogeneous species with a S_{obs} of about 11.5 and 9 S, respectively (Figure 5) [7, 29]. Very similar sedimentation behavior was observed for CcO in bicelles. Enzyme is homogeneous in bicelles with the sedimentation coefficient almost exactly the same as monomeric CcO solubilized in high concentration of TX-100 (Figure 5). However, as it was stated above the sedimentation coefficient for monomeric enzyme solubilized in DM is ~ 11.5 S. Therefore, based on sedimentation velocity results it's too speculative to make a conclusion about the aggregation state of CcO in bicelles, but our data clearly demonstrate presence of only one homogeneous population of "proteobicelles".

Thermal denaturation of CcO incorporated into bicelles – DSC and enzymatic activity analysis

Very recently, Sedláč et al. [45] showed that aggregation state of detergent-solubilized CcO can be estimated using thermal denaturation data obtained by DSC. We have compared the apparent transition temperatures (T_{trs}) of DSC runs performed in conditions inducing either monomeric or dimeric state of CcO with that measured in bicelles. Our results obtained in 14 mM DM with or without cholate are very similar to that published by Sedláč et al. [45]. Thermal denaturation of bovine CcO consisted of two consecutive irreversible steps (Figure 6). DSC recorded thermal denaturation of CcO in DMPC/DHPC mixture exhibits also two-step transition (Figure 6). Midpoint of the first transition (46.9 °C) agrees well with that performed for monomeric CcO in 14 mM DM (47.2 °C). This is about 2.5 °C lower than T_{trs} value determined for partially dimeric CcO in 14 mM DM plus 2 mM sodium cholate and >4 °C lower than T_{trs} for dimeric CcO in 2 mM DM and 2 mM sodium cholate, indicating that CcO in DMPC/DHPC bicelles is in monomeric form. Distinction in T_{trs} of the second transition as well as its asymmetry could be explained by the nature of the CcO binding in the bicelles or by stabilizing effect of phospholipids. Values of T_{trs} and calorimetric enthalpies related to CcO thermal denaturation are summarized in Table 2. It was also shown by Sedláč et al. [45] that the transition temperature of the first step is sensitive to structural features of the protein, such as presence or absence of bound phospholipids or

oligomeric (aggregation) state. The effect of increasing of DM concentration, specifically from 2 mM to 14 mM, was accompanied by the decrease of T_{trs} from 51.6 °C to 48.0 °C [45]. However, the presence of 2 mM sodium cholate has a stabilization effect on the first CcO thermal transition as demonstrated by an increase in T_{trs} roughly by 2 °C over the range of 2 to 14 mM DM [45]. Addition of sodium cholate to DM or TX-100 solubilized CcO induces dimerization of enzyme as was revealed by sedimentation analysis [7]. Therefore, the observed stabilization is caused most probably by the CcO dimer formation since two of the four dissociable subunits form interface within the two 13-subunit monomers. T_{trs} value of the second step is almost unaffected by the change of CcO oligomeric state (resulting from the presence of the two mentioned detergents) although destabilization caused by removal of tightly bound cardiolipins has revealed the T_{trs} value drops by almost 4 °C [45].

Temperature-induced functional modification of CcO was investigated by monitoring electron transfer activity of the enzyme after it had been heated to a particular temperature. The initial experimental conditions were: 5 μ M CcO in 25 mM phosphate buffer containing either, 2 mM DM or 24 mM (C_L) bicelles with a heating rate of 1.5 °C/min. At particular temperature an aliquot was removed, cooled on ice, and the enzymatic activity was subsequently measured at 25 °C. DM-solubilized CcO activity decreased with increasing temperature in a sigmoidal way with a midpoint at ~ 52 °C (Figure 7) indicating that the loss of activity is most likely associated with the first DSC thermal transition at ~ 49.7–54.3 °C observed for dimeric CcO (Table 2). These experimental data are in excellent agreement with temperature-induced inactivation of bovine heart CcO published recently [45]. However, although the activity of CcO in bicelles decreases with increasing temperature by a similar a sigmoidal way, a midpoint was at ~ 45 °C indicating close association with the first DSC thermal transition at ~ 47.2 °C which was observed for monomeric CcO (Figure 7, Table 2). Both, DSC and temperature-induced activity changes of CcO suggests, that CcO in bicelles is monomeric. Although indirectly, but this suggestion is also supported by sedimentation velocity data. The ability of bicelles to induce monomerization is somewhat unexpected. It was demonstrated that the cholate/phospholipid or cholate/nonionic detergent mixed micelles favor CcO dimerization [7]. Isolated in cholate preparation A of CcO is always co-purified with 2–10 molecules of sodium cholate bound to specific protein sites [2]. Moreover such isolated enzyme is solubilized in buffer containing sodium cholate (refer to Methods section). Therefore, in our experiment adding of sodium cholate-solubilized CcO to bicelles set up the conditions for enzyme dimerization. A contrary effect, however, was observed. The most likely explanation that bicelles induce CcO monomerization is interaction of protein with detergent-like DHPC. We believe that short-chain DHPC acts the same way as non-ionic detergent micelles, such as TX-100 or DM, which at specific conditions induce CcO monomerization [7, 29]. As a result, DHPC structurally rearrange or replace detergent or phospholipid located at the protein surface, thereby it prohibits the necessary protein-protein contacts.

Conclusions

In conclusion, in this work for the first time phospholipid bicelles were used for studying mitochondrial electron transfer Complex IV or cytochrome *c* oxidase. A protocol described in the present work includes preparation of bicelles from DMPC and DHPC mixture at total

concentration of phospholipids $C_L = 24$ mM (12%, w/v) and the molar ratio q equal to 0.4, and successful incorporation of CcO isolated from bovine heart into bicelles. Both, sedimentation velocity analysis and temperature-induced denaturation suggested that enzyme in bicelles is monomeric. In support to recently published results by Sedlák et al. [45] our data indicate that thermal denaturation analysis can be used as a tool for determination of CcO aggregation state. Characterization of both bicelles and proteobicelles using absorption spectroscopy, dynamic light scattering, atomic force microscopy, sedimentation velocity and differential scanning calorimetry demonstrated that bicelles introduce perhaps a unique opportunity to study mitochondrial proteins in a milieu relatively unchanged (bilayer) from their physiological environment.

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Abbreviations

CcO	cytochrome <i>c</i> oxidase
DMPC	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphocholine
DHPC	1,2-dihexanoyl- <i>sn</i> -glycero-3-phosphocholine
PL	phospholipids
C_L	total molar concentration of phospholipids in bicelles
q	molar ratio of long-chain DMPC over short-chain DHPC
EDTA	ethylenediaminetetraacetic acid
DM	n-Dodecyl β -D-maltoside
TX-100	Triton X-100
AFM	atomic force microscopy
DSC	differential scanning calorimetry
DLS	dynamic light scattering

References

1. Wikström MKF. Proton pump coupled to cytochrome *c* oxidase in mitochondria. *Nature*. 1977; 266:271–273. [PubMed: 15223]
2. Yoshikawa S, Shimada A. Reaction mechanism of cytochrome *c* oxidase. *Chem Rev*. 2015; 115:1936–1989.10.1021/cr500266a [PubMed: 25603498]
3. Rais I, Karas M, Schägger H. Two-dimensional electrophoresis for the isolation of integral membrane proteins and mass spectrometric identification. *Proteomics*. 2004; 4:2567–2571. [PubMed: 15352231]
4. Fandino AS, Rais I, Vollmer M, Elgass H, Schägger H. LC-nanospray-MS/MS analysis of hydrophobic proteins from membrane protein complexes isolated by blue-native electrophoresis. *J Mass Spectrom*. 2005; 40:1223–1231. [PubMed: 16127664]

5. Robinson NC, Capaldi RA. Interaction of detergents with cytochrome c oxidase. *Biochemistry*. 1977; 16:375–381. [PubMed: 189798]
6. Garavito MR, Fergusson-Miller S. Detergents as tools in membrane biochemistry. *J Biol Chem*. 2001; 276:32403–32406.10.1074/jbc.R100031200 [PubMed: 11432878]
7. Musatov A, Robinson NC. Cholate induces dimerization of detergent or phospholipid solubilized cytochrome c oxidase. *Biochemistry*. 2002; 41:4371–4376. [PubMed: 11914083]
8. Wilson KS, Prochaska LJ. Phospholipid vesicles containing bovine heart mitochondrial cytochrome c oxidase and subunit III-deficient enzyme: analysis of respiratory control and proton translocating activities. *Arch Biochem Biophys*. 1990; 292:413–420. [PubMed: 2173485]
9. Wikström MKF, Saari HT. The mechanism of energy conservation and transduction by mitochondrial cytochrome c oxidase. *Biochim Biophys Acta*. 1977; 462:347–361. [PubMed: 201286]
10. Vygodina TV, Zakirzianova W, Konstantinov AA. Inhibition of membrane-bound cytochrome c oxidase by zinc ions: High-affinity Zn²⁺-binding site at the P-side of the membrane. *FEBS Lett*. 2008; 582:4158–4162. [PubMed: 19041645]
11. Öjemyr LN, von Ballmoos C, Gennis RB, Sligar SG, Brzezinski P. Reconstitution of respiratory oxidases in membrane nanodiscs for investigation of proton-coupled electron transfer. *FEBS Lett*. 2012; 586:640–645. [PubMed: 22209982]
12. Sanders SR, Prestegard JH. Magnetically orientable phospholipid bilayers containing small amounts of a bile salt analogue, CHAPSO. *Biophys J*. 1990; 58:447–460. [PubMed: 2207249]
13. Dürr UHN, Gildenberg M, Ramamoorthy A. The magic of bicelles lights up membrane protein structure. *Chem Rev*. 2012; 112:6054–6074. [PubMed: 22920148]
14. Brindley AJ, Martin RW. Effect of divalent cations on DMPC/DHPC bicelle formation and alignment. *Langmuir*. 2012; 28:7788–7796. [PubMed: 22548306]
15. Glover KJ, Whiles JA, Wu G, Yu N, Deems R, Struppe JO, Stark RE, Komives EA, Vold RR. Structural evaluation of phospholipid bicelles for solution-state studies of membrane-associated biomolecules. *Biophys J*. 2001; 81:2163–2171. [PubMed: 11566787]
16. Arnold A, Labrot T, Oda R, Dufourc EJ. Cation modulation of bicelle size and magnetic alignment as revealed by solid-state NMR and electron microscopy. *Biophys J*. 2002; 83:2667–2680. [PubMed: 12414699]
17. Marcotte I, Auger M. Bicelles as model membranes for solid and solution-state NMR studies of membrane peptides and proteins. *Concept Magn Reson A*. 2005; 24A:13–18.
18. Ujwal R, Bowie JU. Crystallizing membrane proteins using lipidic bicelles. *Methods*. 2011; 55:337–341. [PubMed: 21982781]
19. Bárány-Wallje E, Andersson A, Gräslund A, Mäler L. NMR solution structure and position of transportin in neutral phospholipid bicelles. *FEBS Lett*. 2004; 567:265–269. [PubMed: 15178334]
20. Nieh MP, Glinka CJ, Krueger S, Prosser RS, Katsaras J. SANS study of the structural phases of magnetically alignable lanthanide-doped phospholipid mixtures. *Langmuir*. 2001; 17:2629–2638.
21. Fowler LR, Richardson SH, Hatefi Y. A rapid method for the preparation of highly purified cytochrome oxidase. *Biochim Biophys Acta*. 1962; 64:170–173. [PubMed: 13958990]
22. Capaldi RA, Hayashi H. The polypeptide composition of cytochrome oxidase from beef heart mitochondria. *FEBS Lett*. 1972; 26:261–263. [PubMed: 4344291]
23. Sedlák E, Robinson NC. Phospholipase A₂ digestion of cardiolipin bound to bovine cytochrome c oxidase alters both activity and quaternary structure. *Biochemistry*. 1999; 38:14966–14972. [PubMed: 10555978]
24. Dale MP, Robinson NC. Synthesis of cardiolipin derivatives with protection of the free hydroxyl: its application to the study of cardiolipin stimulation of cytochrome c oxidase. *Biochemistry*. 1988; 27:8270–8275. [PubMed: 2852959]
25. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959; 37:911–917. [PubMed: 13671378]
26. Soulimane T, Buse G. Integral cytochrome-c oxidase: preparation and progress towards a three-dimensional crystallization. *Eur J Biochem*. 1995; 227:588–595. [PubMed: 7851442]

27. Fabian M, Palmer G. Hydrogen peroxide is not released following reaction of cyanide with several catalytically important derivatives of cytochrome *c* oxidase. *FEBS Lett.* 1998; 422:1–4. [PubMed: 9475157]
28. van Holde KE, Weischet WO. Boundary analysis of sedimentation-velocity experiments with monodisperse and paucidisperse solutes. *Biopolymers.* 1978; 17:1387–1403.
29. Musatov A, Ortega-Lopez J, Robinson NC. Detergent-solubilized bovine cytochrome *c* oxidase: dimerization depends on the amphiphilic environment. *Biochemistry.* 2000; 39:12996–13004. [PubMed: 11041865]
30. Nagle JF, Tristam-Nagle S. Structure of lipid bilayers. *Biochim Biophys Acta.* 2000; 1469:159. [PubMed: 11063882]
31. Van Dam L, Karlsson G, Edwards K. Direct observation and characterization of DMPC/DHPC aggregates under conditions relevant for biological solution NMR. *Biochim Biophys Acta.* 2004; 1664:241–256. [PubMed: 15328057]
32. Wu H, Su K, Guan X, Sublette ME, Stark RE. Assessing the size, stability, and utility of isotropically tumbling bicelle systems for structural biology. *Biochim Biophys Acta.* 2010; 1798:482–488. [PubMed: 19914202]
33. Harroun TA, Koslowsky M, Nieh MP, de Lannoy CF, Raghunathan VA, Katsaras J. Comprehensive examination of mesophases formed by DMPC and DHPC mixtures. *Langmuir.* 2005; 21:5356–5361. [PubMed: 15924461]
34. Robinson NC, Talbert L. Triton X-100 induced dissociation of beef heart cytochrome *c* oxidase into monomers. *Biochemistry.* 1986; 25:2328–2335. [PubMed: 3013301]
35. Panda M, Robinson NC. Kinetics and mechanism for the binding of HCN to cytochrome *c* oxidase. *Biochemistry.* 1995; 34:10009–10018. [PubMed: 7632673]
36. Vygodina T, Konstantinov AA. Effect of pH on the spectrum of cytochrome *c* oxidase hydrogen peroxide complex. *Biochim Biophys Acta.* 1989; 973:390–398. [PubMed: 2538152]
37. Fabian M, Palmer G. The interaction of cytochrome oxidase with hydrogen peroxide: the relationship of compound P and compound F. *Biochemistry.* 1995; 34:13802–13810. [PubMed: 7577973]
38. Baker GM, Noguchi M, Palmer G. The reaction of cytochrome oxidase with cyanide. Preparation of the rapidly reacting form and its conversion to the slowly reacting form. *J Biol Chem.* 1987; 262:595–604. [PubMed: 3027057]
39. Fabian M, Skultety L, Brunel C, Palmer G. Cyanide Stimulated Dissociation of Chloride from the Catalytic Center of Oxidized Cytochrome *c* Oxidase. *Biochemistry.* 2001:6061–6069. [PubMed: 11352742]
40. Musatov A, Konstantinov AA. Conformational change of cytochrome *a*₃ induced by oxidized cytochrome *c*. *FEBS Lett.* 1988; 238:295–299. [PubMed: 2844603]
41. Morgan JE, Verchovsky MI, Palmer G, Wikström MKF. Role of the PR intermediate in the reaction of cytochrome *c* oxidase with O₂. *Biochemistry.* 2001; 40:6882–6892. [PubMed: 11389603]
42. Mahapatro SN, Robinson NC. Effect of changing the detergent bound to bovine cytochrome *c* oxidase upon its individual electron transfer steps. *Biochemistry.* 1990; 29:764–770. [PubMed: 2159789]
43. Antalík M, Jancura D, Palmer G, Fabian M. Role for the protein in internal electron transfer to the catalytic center of cytochrome *c* oxidase. *Biochemistry.* 2005; 44:14881–14889. [PubMed: 16274235]
44. Vik SB, Capaldi RA. Lipid requirements for cytochrome *c* oxidase activity. *Biochemistry.* 1977; 16:5755–5759. [PubMed: 201276]
45. Sedlák E, Varha R, Musatov A, Robinson NC. The kinetic stability of cytochrome *c* oxidase: effect of bound phospholipid and dimerization. *Biophys J.* 2014; 107:2932–2940. [PubMed: 25517158]

Research highlights

- The protocol for incorporation of cytochrome c oxidase into bicelles was described
- Cytochrome c oxidase in bicelles remains structurally and functionally intact
- Most likely cytochrome c oxidase is monomeric in bicelles
- Bicelles are promising membrane model structures for investigations of electron transfer complexes

Figure 1. Comparison of the optical spectra of preparation A of CcO solubilized in dodecyl maltoside (left panel) with CcO in DHPC (middle panel) and DMPC/DHPC bicelles (right panel). Spectra of 5.1 μ M oxidized CcO (solid lines) were taken after solubilization of enzyme in 20 mM Tris-Cl buffer, pH 7.4 containing either 2 mM DM (left panel), 33 mM DHPC or 24 mM DMPC/DHPC mixture (right panel). Reduced CcO (dashed lines) was obtained by addition of a few crystals of dithionite to oxidized enzyme.

Figure 2. Atomic force microscopy

(A) AFM imaging of bicelles prepared from 17 mM DHPC and 7 mM DMPC in 20 mM phosphate buffer, pH 7.4. The xy scale is 5×5 μm. (B) Bicelles heights determination. The panel is perpendicular to the scanning plane view of the upper image. (C) Zoomed 2.5×2.5 μm area selected and marked by red square in image A. (D) Representative data of determination of bicelles diameter and height. Surface profiling (or cross-section) lines were drawn across the part of the AFM image between two bicelles marked by arrows with the same color. Data demonstrate the diameter (~ 50 nm) and height (4±1 nm) of the most abundant bicelles. The data analyses were performed using NanoScope Analysis1.20. software.

Figure 3. The apparent hydrodynamic size distribution of bicelles (panel A) and bicelles with CcO isolated in TX-100 (panel B) measured by dynamic light scattering in 20 mM phosphate buffer, pH 7.4 at 25 °C

Bicelles and CcO in bicelles were prepared as described in Materials and Methods. The experimental conditions were: PL final concentration $C_L = 2.4$ mM and CcO = 0.5 μ M.

Figure 4. Spectral changes of preparation A of CcO induced by hydrogen peroxide

Main panel: Absolute spectra before (solid line) and after reaction of CcO in bicelles with 5 mM H_2O_2 (dashed line) for 3 min at 25 °C. Inset panel: Difference spectrum of CcO in bicelles and in DM. CcO in bicelles (red color) and CcO in DM (reacted with H_2O_2 minus untreated CcO). Buffer: 50 mM Tris-Cl buffer, pH 7.4 containing either, DMPC/DHPC bicelles ($C_L = 24$ mM) or 2 mM DM. The concentration of CcO was 5 μM and 4.2 μM in bicelles and DM, respectively.

Figure 5. Sedimentation velocity analysis

The distribution of sedimentation coefficients was determined after solubilization of 5 μ M CcO in 20 mM Tris-Cl buffer, pH 7.4, containing either 10 mM Triton X-100 (blue triangles), 10 mM DM (black circles) or bicelles (DMPC/DHPC, red circles, $C_L = 24$ mM, $q = 0.4$). In each experiment, CcO concentration as a function of radial position was measured by absorbance at 420 nm. Data were collected at 10–12 min intervals during centrifugation of the sample at 40 000 rpm and 20 $^{\circ}$ C.

Figure 6. DSC analysis of CcO (preparation A)

Normalized DSC thermograms of CcO in 20 mM Tris-Cl, pH 8.0 buffer containing either 14 mM DM (blue line), 14 mM DM + 2 mM sodium cholate (black dashed line) or 24 mM mixture of DMPC/DHPC (red line). Concentration of CcO was always 5 μ M. All DSC data were acquired at a scan rate of 1.5 °C/min.

Figure 7. Temperature effect on CcO electron transfer activity

CcO (5 μ M of preparation A) was solubilized in 25 mM phosphate buffer, pH 7.2, containing either 2 mM dodecyl maltoside (circles) or 24 mM DMPC/DHPC bicelles (triangles) in the same buffer. The error in determination of molecular activity was $\pm 5\%$. Solid and dashed lines are sigmoidal non-linear regression fits for DM- and bicelles solubilized CcO, respectively.

Table 1Electron transfer activity of cytochrome *c* oxidase

	Activity was measured in buffer containing 2 mM DM	Activity was measured in buffer containing DMPC/DHPC bicelles ($C_L = 24$ mM)
Dodecyl maltoside solubilized CcO	350 s^{-1} (100%)	90 s^{-1} (30%)
CcO in bicelles	335 s^{-1} (95%)	70 s^{-1} (20%)

In each experiment 5 μM CcO (preparation A) was first solubilized in 25 mM phosphate buffer, pH 7.2, containing either 2 mM dodecyl maltoside or DMPC/DHPC ($C_L = 24$ mM) bicelles. The reaction was always started with an addition of CcO (final concentration = 2 nM) to 30 μM ferrocyanide *c*. In each case electron transfer activity was measured spectrophotometrically at 550 nm. The activity of CcO solubilized and analyzed in 25 mM phosphate buffer, pH 7.2, containing 2 mM DM was $\sim 350\text{ s}^{-1}$ (100%). The error in determination of molecular activity was $\pm 5\%$. Temperature = 25 $^{\circ}\text{C}$.

Table 2

DSC values of transition temperatures and calorimetric enthalpy changes for detergent-solubilized CcO and CcO in bicelles¹

	T_{trs1} (°C)	H₁ (kJ/mol)	T_{trs2} (°C)	H₂ (kJ/mol)
2 mM DM + 2 mM Na-cholate ²	54.3 ± 0.1	280 ± 11	61.1 ± 0.1	1079 ± 15
14 mM DM	47.2 ± 0.1	555 ± 13	60.1 ± 0.1	1071 ± 13
14 mM DM + 2 mM Na-cholate	49.7 ± 0.1	562 ± 8	60.2 ± 0.1	920 ± 7
DMPC/DHPC bicelles	46.9 ± 0.1	527 ± 15	62.2 ± 0.2	1647 ± 220

¹DSC experiments were performed in 20 mM Tris-Cl buffer, pH 8.0 at scan rate of 1.5 °C/min and 5 μM CcO.

²Sedláč et al. Biophys. J. 107 (2014) 2932–2940.